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PATENT
Attorney Docket No. 80129-000100US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of:

JEREMY S. LEE et al.

Application No.: 10/061,979

Filed: January 31, 2002

For: BIOLOGICALLY ACTIVE METAL-
CONTAINING NUCLEIC ACIDS

Examiner: M. Marvich

Art Unit: 1636

RULE 132 DECLARATION

Assistant Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Dr. Sylvia van Drunen Littel-van den Hurk, declare as follows:

1. I have a Ph.D. in Veterinary Microbiology and am presently Senior Scientist
and Program Manager, Vaccine and Infectious Disease Organization, University of
Saskatchewan, Saskatoon, Saskatchewan, Canada. A copy of my C.V. is attached hereto.

2. I conducted experiments which demonstrate that immunization with metal-
complexed DNA (M-DNA) which encodes an antigenic protein elicits an immune response in

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the host animals. These experiments, described below, were conducted by me or under my supervision.

3. The experiments were conducted using an eucaryotic expression vector which contained the tgD gene from bovine herpes virus-1 (Pontarollo, R., Babiuk, L.A., Hecker, R. and van Drunen Littel-van den Hurk, S. 2002. Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG enhanced plasmids. J. Gen. Virol. 83: 2973-2981). The tgD gene encodes a viral surface glycoprotein.

4. The experiments employed a Bio-Rad Helios Gene Gun. The operation of the gene gun and the making of the "bullets" followed procedures as outlined by the manufacturer. The M- and B-DNA for the bullets were made as follows: For normal "B-DNA"(non-metal containing), 100 µg/ml of plasmid comprising the eucaryotic expression vector containing the tgD gene from bovine Herpes virus were incubated at 37°C for 2 hours in a buffer of 10 mM Tris-HCl, 10 mM NaCl pH 8.5. For Zn M-DNA the buffer was supplemented with 20 mM ZnCl₂, and for Ni M-DNA, the buffer was supplemented with 20 mM NiCl₂. To 100 µl of the plasmid DNA prepared as described, 12.5 mg of gold particles was added. To prepare the Zn M-DNA, Ni M-DNA, and B-DNA, 100 µL of 1M ZnCl₂, 100 µl of 500 mM NiCl₂, and 100 µl of CaCl₂, respectively, was added. Then 100 µL of 50 mM spermidine was added and the preparation was allowed to stand at room temp. for 15 min. The preparation was then centrifuged at 5000 rpm (microfuge) for 15 sec and washed with absolute ethanol 4 times, then

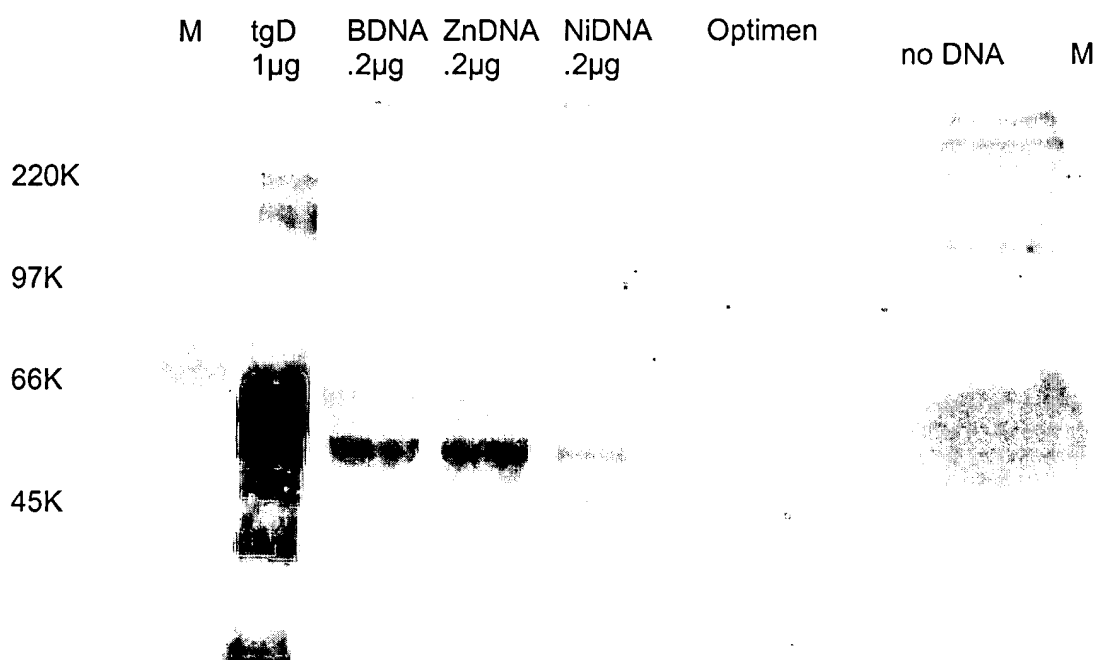
the final pellet was resuspended in 3 ml of polyvinylpyrrolidone (PVP). Bullets for use in the gene gun were prepared according to instructions in the BioRAD manual.

5. Protein expression by the M-DNA constructs was confirmed by Western blot of tissue culture supernatants. Six-well tissue culture plates of Cos-7 cells in DMEM + 10% FBS were grown overnight at 37°C in 5%CO₂. The next afternoon, the media was gently removed and the plate covered with parafilm having holes cut in the center of each well of the plate to prevent cross-contamination when shooting the gene gun. The gene gun was then shot at a pressure of 200psi. Two ml of Optimem serum-free media (Invitrogen) was added to the wells and the plate incubated for 48 hours. After 48 hrs. the supernatant was collected and concentrated from 2ml to 200µl using a 4ml. Millipore spin concentrator. Twenty-five microliters of a mixture of protease inhibitors was added to the supernatant, and samples were frozen at -20°C until ready for use on SDS-PAGE gel. To extract protein from cells, cells were scraped into 250µl of RIPA buffer (0.01M Tris-HCl pH 7.5, 0.15M NaCl, 1% (w/v) sodium deoxycholate, 1% (v/v) NP40) with 25µl of protease inhibitors, and samples were frozen at -20°C. Samples were run on 8.5% SDS Polyacrylamide gel using 1.5mm spacers, transferred to nitrocellulose membrane and Western blotting performed using a murine monoclonal antibody to tgD.

6. The Western blot of protein extracted from Cos-7 cells transfected with the tgD expression plasmid via the gene gun is shown below. M=molecular weight markers; tgD=

control isolated protein. The band at about 50K clearly indicates that the tgD protein has been expressed in the cells treated with B-DNA as well as in the cells treated with Zn or Ni M-DNA.

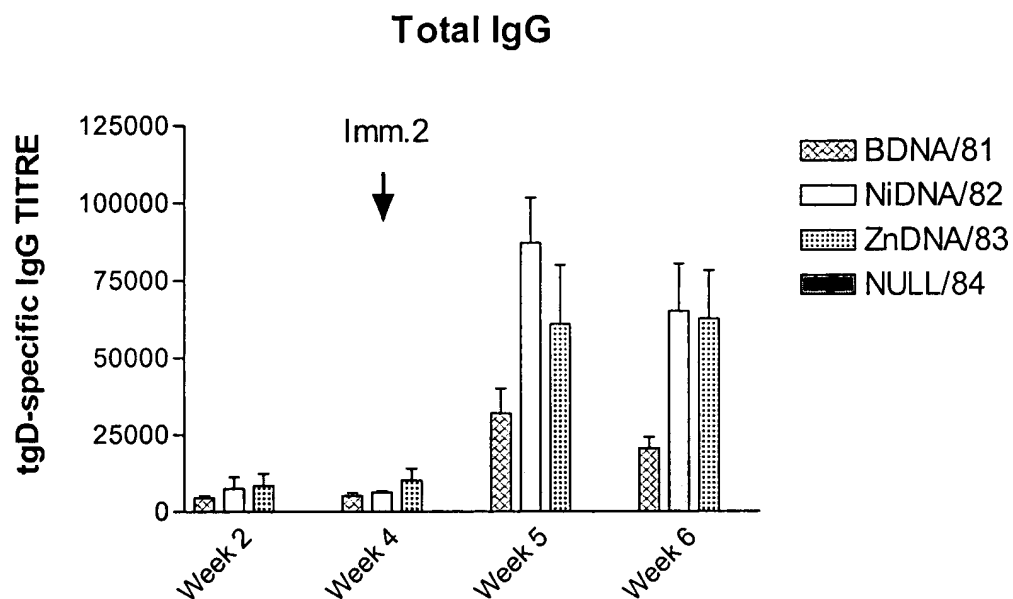
Western blot of protein extracted from Cos-7 cells transfected with the tgD



7. To demonstrate that the M-DNA encoded a protein which could be transcribed and expressed as an immunogenic protein in animals, mice were immunized with the plasmid encoding the tgD herpes virus surface glycoprotein described above. Mice (n = 5 per group) were vaccinated on day 1 and boosted on day 28 using the Gene Gun, with 0.2 ug of plasmid encoding tgD either as B-DNA, or as NiM-DNA or ZnM-DNA.

The mice were bled on days 35 and 42 and the serum was tested by standard ELISA for the presence of antibodies specific for the tgD glycoprotein using purified tgD antigen on the plates.

The results are illustrated below.




The ELISA results (above) clearly show that the mice immunized with either Ni or Zn M-DNA produce a sustained antibody response by weeks 5 and 6 which is 2-3 fold higher than that for B-DNA. These results confirm that M-DNA can be used in animals to express antigenic proteins, and that the DNA transcribes normally to produce an immune response that recognizes the native antigen.

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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed: _____



Dr. Sylvia van Drunen Littel-van den Hurk

Dated: _____

Sept 10/07



CURRICULUM VITAE

Sylvia van Drunen Littel-van den Hurk

Senior Scientist and Program Manager
Vaccine and Infectious Disease Organization
University of Saskatchewan
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SK S7N 5E3, Canada
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EMPLOYMENT

1995-pres. Senior Scientist and Program Manager. Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, SK.
1990-1995 Research Scientist. Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, SK.
1985-1990 Professional Research Associate. Veterinary Infectious Disease Organization, University of Saskatchewan, Saskatoon, SK.
1984-1985 Research Associate. Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK.
1978-1981 Research Assistant. Cancer Centre, McGill University, Montreal, QC.
1975-1976 Research Assistant. Comparative Virology Group, Faculty of Medicine, University of Montreal, QC.

PROFESSIONAL AFFILIATIONS

American Society of Microbiology, Washington, DC
Canadian Society of Microbiologists, Ottawa, ON.
American Society for Virology
Canadian Vaccines and Therapeutics Network

EDUCATION

Ph.D. in Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK.
M.Sc. in Molecular Virology, University of Wageningen, Wageningen, Netherlands.
B.Sc. in Plant Pathology, University of Wageningen, Wageningen, Netherlands.

TRI-COUNCIL RESEARCH FUNDING DURING THE PAST FIVE YEARS

2004-2008	Molecular characterization of the pathogenesis of RSV and the effects of CpG adjuvanted RSV vaccines. CIHR \$86,200/year
2001-2005	Characterization of tegument proteins of bovine herpesvirus-1. NSERC Operating Grant. \$37,000/year.
2002-2005	Innovative vaccine development and delivery to induce mucosal immunity. CIHR Health research program of Excellence. Group Grant. \$400,000/year.
2000-2004	CpG motifs as immune adjuvants against respiratory disease. Canadian Institute of Health Research Canada. \$77,474/year.
2000-2003	Enhancement of DNA vaccine efficacy by targeting antigen presenting cells. NSERC Strategic Grant. \$138,000/year.
1997-2001	Characterization of bovine herpesvirus-1 glycoproteins. NSERC Operating Grant. \$37,000/year.

PROVINCIAL RESEARCH FUNDING DURING THE PAST FIVE YEARS

2003-2006	Development of neonatal immunization strategies against bovine respiratory disease using CpG motifs as adjuvants. Alberta Agricultural Research Institute matching grant. \$66,350/year.
2002-2005	Development of neonatal immunization strategies using CpG motifs as immune adjuvants. Agricultural Development Fund Saskatchewan. \$60,300/year.
2000-2003	Development of BVDV DNA vaccines. Ontario Cattlemen's Association. \$17,300/year.
1999-2002	Optimization of gene gun mediated delivery of DNA vaccines. BC Cattle Industry Development Council. \$38,500/year.
1999-2002	Optimization of gene gun mediated delivery of DNA vaccines. Alberta Agricultural Research Institute matching grant. \$104,000/year.
1999-2002	Development of mono- and multivalent DNA vaccine for cattle. Agricultural Development Fund Saskatchewan. \$49,000/year.
1998-2000	Development of DNA-based vaccination methods for cattle. Ontario Cattlemen's Association. \$50,000/year.

PATENTS

- U.S. Patent Nr. 5,951,988. Van Drunen Littel-van den Hurk, S., Zamb, T., and Redmond, M. Adjuvant formulations with enhanced immunogenic activity and related compositions and methods. Issued September 14, 1999.
- European Patent. Nr. 0618814. Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick. Recombinant bovine herpesvirus type-1 polypeptides and vaccines. Issued April 14, 1999.

- U.S. Patent Nr. 5,879,895. Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick. Recombinant bovine herpesvirus type-1 polypeptides and immunoassays. Issued March 9, 1999.
- U.S. Patent Nr. 5,858,989. Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick. Vaccine comprising sequences encoding bovine herpesvirus type 1 gI, gIII and gIV. Issued January 12, 1999.
- U.S. Patent Nr. 5,585,264. Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick, D.R. Nucleotide sequences encoding bovine herpesvirus type-1 gI, gIII and gIV polypeptides. Issued December 17, 1996.
- Canadian Patent Nr. 1,338,069. Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick, D.R. Bovine herpesvirus type-1 polypeptides and vaccines. Issued February 26, 1996.
- U.S. Patent Nr. 5,151,267. Babiuk, L.A., van den Hurk, S., Zamb, T. and Fitzpatrick, D.R. Bovine herpesvirus type-1 polypeptides and vaccines. Issued September 29, 1992.

PUBLICATIONS IN REFEREED JOURNALS DURING THE PAST FIVE YEARS

- Babiuk, S., Mookerjee, N., Pontarollo, R., Griebel, P., van Drunen Littel-van den Hurk, S., Hecker, R. And Babiuk, L.A. 2004. TLR0^{-/-} and TLR9^{+/+} mice elicit similar immune responses to a DNA vaccine. *Immunology. In press*.
- Zheng, C., Brownlie, R., Babiuk, L. A. and van Drunen Littel-van den Hurk, S. 2004. Characterization of nuclear Localization and Export Signals of the Major Tegument Protein VP8 of Bovine Herpesvirus-1. *Virology* **324**: 327-339.
- Manoj, S., Babiuk, L.A., Karvonen, B.C. and van Drunen Littel-van den Hurk, S. 2004. Modulation of immune responses to bovine herpesvirus-1 in cattle by immunization with a DNA vaccine encoding glycoprotein D as a fusion protein with bovine CD154. *Immunology* **112**: 328-338.
- Yu, H, Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2004. Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3. *J. Gen. Virol.* **85**: 1533-1543.
- Manoj, S., Babiuk, L.A. and Drunen Littel-van den Hurk, S. 2003. Immunization with a dicistronic plasmid expressing a truncated form of bovine herpesvirus-1 glycoprotein D and the amino terminal subunit of glycoprotein B results in reduced gB-specific immune responses. *Virology* **313**:296-307.
- Ioannou, X.P., Griebel, Mena, P.A., Gomis, S.M., Godson, D.L., Mutwiri, G., Hecker, R., Babiuk L.A. and S. van Drunen Littel-van den Hurk, S. 2003. Safety of CpG oligodeoxynucleotides in veterinary species. *Antisense and Nucleic Acid Drug development.* **13**:157-161.
- Ioannou, X., Gomis, S.M., B. Karvonen, B., Thrush, T., R. Hecker, R., L. A. Babiuk, L.A. and van Drunen Littel-van den Hurk S. 2003. Oligodeoxynucleotides containing CpG motifs are safe and effective adjuvants for rabbits. *Vaccine* **21**:127-37.
- Manoj, S., Griebel, P.J., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2003. Targeting with bovine CD154 enhances humoral immune responses induced by a DNA vaccine in sheep. *J. Immunol.* **170**: 989-996.

- Ioannou, X., Gomis, S.M., Karvonen, B., Hecker, R., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2002. Formulation of CpG-containing oligodeoxynucleotides with conventional adjuvants enhances the magnitude and changes the bias of the immune responses to a herpesvirus glycoprotein. *Vaccine* 21:127-37.
- Pontarollo, R., Babiuk, L.A., Hecker, R. and van Drunen Littel-van den Hurk, S. 2002. Augmentation of cellular immune responses to bovine herpesvirus-1 glycoprotein D by vaccination with CpG enhanced plasmids. *J. Gen. Virol.* 83: 2973-2981.
- Ioannou, X., Babiuk, P., Griebel, L.A., Hecker, R. and van Drunen Littel-van den Hurk, S. 2002. The immunogenicity and protective efficacy of bovine herpesvirus-1 glycoprotein D are increased by formulation with CpG oligonucleotides. *J. Virol.* 76: 9002-9010.
- Rankin, R., Pontarollo, R., Gomis, S., Karvonen, B., Willson, P., Loehr, B.I., Godson, D., Hecker, R., Babiuk, L.A., and van Drunen Littel-van den Hurk, S. 2002. CpG-containing oligodinucleotides augment and switch the immune responses of cattle to glycoprotein D of bovine herpesvirus-1. *Vaccine* 20: 3014-3022.
- Mena, A., Ioannou, X.P., van Kessel, A., van Drunen Littel-van den Hurk, S., Popowych, Y., Babiuk, L.A. and Godson, D.L. 2002. Th1/Th2 biasing effects of vaccination oin cattle as determined by real-time PCR. *J. Imm. Methods.* 263: 11-21.
- Pontarollo, R., Rankin, R., Godson, D., Griebel, P., Krieg, A.M., Hecker, R., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2002. Monocytes are required for optimum stimulation of bovine peripheral blood mononuclear cells by non-methylated CpG motifs. *Vet. Immunol. Immunopathol.* 84: 43-59.
- Suradhat, S., Braun, R. P., Lewis, P. J., Babiuk, L. A., van Drunen Littel-van den Hurk, S., Griebel, P. J. and Baca-Estrada, M. E. 2001. Immunization with plasmids encoding antigen-complement C3d chimeras inhibits antigen-specific immune responses. *Veterinary Immunol. Immunopathol.* 83:79-92I.
- Loehr, B.I., Karvonen, B., Willson, P., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2001. Priming by DNA immunization augments T-cell responses to modified live herpesvirus vaccine. *J. Gen. Virol.* 82: 3035-3043.
- Loehr, B I., Rankin, R. Pontarollo, R., King, T., Willson, P., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2001. Suppository mediated DNA immunization induces mucosal immunity against herpesvirus infection in cattle. *Virology* 289:327-333.
- Rankin, R. Pontarollo, R. Ioannou, X., Krieg, A.M., Hecker, R., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2001. Identification of a stimulatory CpG motif for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense and Nucleic Acid Drug development.* 11: 333- 340.
- Van Drunen Littel-van den Hurk, S, Myers, D., Doig, P.A., Karvonen, B., Habermehl, M., Babiuk, L.A., Jelinski, M., Van Donkersgoed, J., Schlesinger K. and Rinehart, C. 2001. Identification of a mutant bovine herpesvirus-1 (BHV-1) in post-arrival outbreaks of IBR in feedlot calves and protection with conventional vaccination. *Can. J. Vet. Res.* 65: 81-88.
- Connolly, S.A., Whitbeck, J.C., Rux, A.H., Krummenacher, C., van Drunen Littel-van den Hurk, S., Cohen, G.H. and Eisenberg, R.J. 2001. Glycoprotein D homologues in herpes simplex virus type-1, pseudorabies virus, and bovine herpesvirus type-1 bind directly to human HveC (Nectin-1) with different affinities. *Virology* 280: 7-18.

- Uwiera, R.R., Rankin, R., Adams, G.P., Pontarollo, R.A., van Drunen Littel-van den Hurk, S., Middleton, D.M., Babiuk, L.A. and Griebel, P.J. 2001. Effects of intradermally administered plasmid deoxyribonucleic acid on ovine popliteal lymph node morphology. *Anatomical Record*. 262:186-192.
- Deregt, D., Jordan, L.T., van Drunen Littel-van den Hurk, S., Masri, S., Tessaro, S.V. and Gilbert, S.A. 2000. A genital herpesvirus isolated from a North-American elk is a unique virus antigenically related to bovine herpesvirus-1. *American J. Vet. Res.* 61: 1614-1618.
- Gerdts, V. Babiuk, L.A., van Drunen Littel-van den Hurk, S. and Griebel, P.J. 2000. Fetal immunization by a DNA vaccine delivered in the amniotic fluid. *Nature Medicine* 6: 929-939.
- Loehr, B.I., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2000. Gene gun mediated DNA immunization primes the development of mucosal immunity against bovine herpesvirus-1 in cattle. *J. Virol.*74: 6077-6086.

Eighty-eight publications in refereed journals in total.

BOOK CHAPTERS AND REVIEW ARTICLES DURING THE PAST FIVE YEARS

- Van Drunen Littel-van den Hurk, S., Babiuk, S. and Babiuk, L.A. 2004. Strategies for Improved Formulation and Delivery of DNA Vaccines to Veterinary Target Species. In. *Immunological reviews: DNA vaccines and Adjuvants*. In press.
- Manoj, S., Babiuk, L.A. and van Drunen Littel-van den Hurk, S. 2004. Approaches for enhancing the efficacy of DNA vaccines' *Critical Reviews in Clinical Laboratory Sciences*. In press.
- Babiuk, L.A., Pontarollo, R., Babiuk, S., Loehr, B. and van Drunen Littel-van den Hurk, S. 2003. Induction of immune responses by DNA vaccines in large animals. *Vaccine* 21:649-658.
- Mutwiri, G., Pontarollo, R., Babiuk, S., Griebel, P., van Drunen Littel-van den Hurk, S., Mena, A., Tsang, C., Alcon, V., Nichani, A., Ioannou, X., Gomis, S., Townsend, H., Hecker, R. Potter, A., and Babiuk, L.A.. 2002. Biological activity of immunostimulatory CpG DNA motifs in domestic animals. *Vet Immunol Immunopathol* 30:89-103.
- Van Drunen Littel-van den Hurk, S., Loehr, B.I. and Babiuk, L.A. 2001. Immunization of livestock with DNA vaccines: current studies and future prospects. *Vaccine* 21:2474-2479.
- Babiuk, L.A., Babiuk, S. Loehr, B.I. and van Drunen Littel-van den Hurk, S. 2000. Nucleic Acid vaccines: Research Tool or Commercial Reality. *Vet. Immunol. Immunopathol.* 76: 1-23.
- Van Drunen Littel-van den Hurk, S., Gerdts, V., Loehr, B.I., Pontarollo, R., Rankin, R., Uwiera, R. and Babiuk, L.A.. 2000. Recent advances for the treatment of diseases of farmed animals. *Advanced drug delivery reviews* 43:13-28.
- Babiuk, L.A., van Drunen Littel-van den Hurk, S., Loehr, B.I. and Uwiera, R. 2000. Veterinary applications of DNA vaccines. *Development and Clinical Progress of DNA vaccines*. Dev. Biol. 104: 73-81. Brown, F., Cichutek, K. and Robertson, J. eds.

Seventeen review articles and bookchapters in total.

RESEARCH AND TECHNICAL REPORTS

- Van Drunen Littel-van den Hurk, S. 2002. Update on Fact Sheet on Infectious Bovine Rhinotracheitis. Alberta Feedlot Management Guide.
- Van Drunen Littel- van den Hurk, S. 2000. Bovine Parainfluenzavirus-3. Animal Health and Production Compendium for CAB International.
- Van Drunen Littel-van den Hurk, S. 1999. Fact Sheet on Infectious Bovine Rhinotracheitis. Alberta Feedlot Management Guide.

MEETINGS ABSTRACTS AND PRESENTATIONS

Eighty-eight abstracts at international scientific meetings.

OTHER PROFESSIONAL ACTIVITIES

- Panel member of the Biomedical Establishment Grant Review Committee of the Saskatchewan Health Research Foundation.
- Ad hoc reviewer for CIHR, NSERC, Alberta Heritage, Biotechnology and Biological Sciences Research Council (BBSRC; UK) and Wellcome Trust (UK).
- Reviewer for J. Virol., Virology, Vaccine, J. Gen. Virol., Viral Immunology, Virus Research, Veterinary Microbiology and Cytokine.
- Lecturing in Advanced Virology, Vaccinology and Recent Advances in Microbiology.

CURRENT RESEARCH PROJECTS AND INTERESTS

- Development of methods for DNA immunization of veterinary species.
- Pathogenesis of bovine herpesvirus-1 and bovine respiratory syncytial virus.
- Immune modulatory effects of CpG motifs.
- Development of hepatitis C virus vaccination strategies.

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